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Detoxification of Aflatoxin B1 using Lactic acid bacteria

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Abstract

Aflatoxin, the mycotoxin is produced by *Aspergillus flavus* and other aflatoxigenic *Aspergillus sp*. These toxins are common contaminant of food and feed, and are posing health hazards to humans and animals alike. Because of the dangerous effects of aflatoxins, detoxification techniques with preventive and remediative measures are necessary. Physical and chemical methods that are followed for decontamination are present are found to be inconvenient. In the present study a biological detoxification strategy using Lactic acid bacterial isolates of curd sample was tested against aflatoxin. The toxigenic strain of *A. flavus* & *A. niger* were isolated from grapes and coconut and used to produce AFB1 in yeast extract sucrose medium. Lactic acid producing bacteria like *S. cremoris*, *S. lactis* and *L. acidophilus* were isolated. The blue fluorescent ring visible around the colonies under UV light indicates the capability of the aflatoxinogenic strains to produce aflatoxin. It was found that LAB strains can tolerate and degrades AFB1 very efficiently within 24 hrs of incubation. Efficient reduction of the toxin quantity was observed by *S.cremoris* & *S.lactis* isolates. Binding affinity of aflatoxin by the studied LAB varied from 56%-94%. Results showed that *S.cremoris* and *S.lactis* had aflatoxin binding ab ility of 94% to the *Aspergillus flavus* isolated from grapes. Also, surface binding of the LAB to *Aspergillus flavus* isolated from coconut is 54% and 90%.

Keywords: Aflatoxin B1, Lactic acid Bacteria, *Aspergillus sp*., HPLC, TLC.

INTRODUCTION

Mycotoxins, the secondary metabolites produced by certain filamentous fungi are responsible to cause a toxic response known as mycotoxicosis on consumption by animals and humans. The most generally known mycotoxins are the aflatoxins. These aflatoxins are usually found when the temperature and humidity are optimum for growth of the mold *A. flavus* and *A. parasiticus*, and the rare *A. nomius*. There are at least 16 structurally related Aflatoxins characterized, and among them, only four i.e. AFB1, AFB2, AFG1, and AFG2 are the most important natural contaminants of crops and other agricultural products [1]. Among the various aflatoxins, aflatoxin B (AFB) is the most potent teratogen, mutagen and hepatocarcinogen, and is classified under Group 1 carcinogen by the International Agency for Research in Cancer (IARC) [2]. AFB1 also possesses immunosuppressive properties [3] and is involved in growth impairment observed in children [4].

To improve the organoleptic properties, safety, nutrition value of cereals, biopreservation technologies are most favourable. These aflatoxins are very stable and cannot be degraded up to 270°C in dry conditions, and they can be converted biologically by metabolism in humans and animals into toxic derivatives, such as epoxide, M_1 or M_2 [5] or less toxic derivatives, such as B2a, by microorganisms. A wide array of organisms, including bacteria, yeasts and fungi has been tested for the biological control of aflatoxin contamination. The probiotic mixtures such as *Lactobacillus sp.* and *Propionibacterium sp.* can be added to the dietary supplements to reduce the bioavailability of aflatoxin in feeds [6].

Generally, Lactic acid bacteria (LAB) are accepted to be safe for use in food by the Food and Agricultural Organization of the United States (FAO) and by the European Food Safety Authority (EFSA) who have granted many species with Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) status, respectively [7]. Since Lactic Acid Bacteria are naturally present in many food systems and found to be a part of the human diet for centuries, they were regarded as a safe organism to consume. *L. rhamnosus* strain was able to detoxify about 80% of aflatoxin B within 60 min of treatment than other strains. Although mechanism of action of LAB on Aflatoxin is not yet clear, it is suggested that a

physical union like an adhesion to bacterial cell wall components Such as polysaccharides and peptidoglycans will be carried on, instead of covalent binding or degradation by bacteria metabolism [8]. Chromatography is one among the most popular methods used to analyze mycotoxins such as aflatoxins. LC, TLC and HPLC are the most used quantitative methods in research and routine analysis of aflatoxins [9]. To enhance the sensitivity of HPLC methods for the determination of aflatoxins some purification and clean-up protocols have been used over the years. The current methods used *Aspergillus* differential medium (ADM) mainly for detection of *A. flavus.* These methods followed the use of specific medium that are used to detect the natural fluorescence of Aflatoxin produced by the growing mycelium followed by the use of complex media [10].

Aflatoxin decontamination by Lactic Acid Bacterial isolates is highly promising; however, this field is still underdeveloped. It is known that toxin binding in Lactic Acid Bacteria is strain dependent. In the present study Aflatoxin removal potential of the Lactic acid bacterial isolates have been analysed by HPLC.

MATERIALS & METHODS

Isolation and identification of fungi

Toxigenic strains of *A. flavus* were isolated from grapes and coconut collected from local market, Madurai (Tamilnadu, India). The strains isolated were cultivated on (24 g/l potato-dextrose broth, 15% (w/v) agar agar (Himedia) at 25°C for seven days and were maintained on potato dextrose agar (PDA) medium slants at 4°C throughout this study. The isolates were identified as *Aspergillus flavus* by both macroscopic and microscopic examination.

Isolation and Bacteriological analysis of the LAB

The sample used in isolating the Lactic acid bacteria was curd. To isolate LAB, MRS (de Man, Rogosa and Sharpe) broth and MRS agar were used. The samples were incubated for a temperature of 37°C for a period of 4 complete days. After the samples were enriched in the liquid medium, they were cultured on the MRS agar plates. The cultures were characterized biochemically through the Catalase test, Gram's staining, Motility, Sugar Acidification tests , Growth on 4.0 and 6.5% NaCl, Growth on 0.3% methylene blue, Temperature tolerance test.

Screening of aflatoxin production

Screening of aflatoxin producing strains were specified by the method using Hara et al. [10] *A. flavus* isolates were plated on Hara media and incubated at 25°C for 7 days in dark. Blue Fluorescent Diffusible zone of aflatoxin was detected under UV light (365 nm) specifies the aflatoxin producing strain.

Preparation and production of Aflatoxin (AFB1) by the fungal isolates

Preparation of AFB1 producing *A. flavus* was grown on yeast extract sucrose (YES) broth for AFB_1 production. The spores of 3-day-old culture of the aflatoxin producing *A. flavus* were inoculated into 50 ml YES broth (2% yeast extract, 15% sucrose) in a 250-ml conical flask and incubated at room temperature without shaking. After 7 days of incubation, AFB1 produced in the medium was extracted twice, using an equal volume of chloroform. In addition, the extracts were concentrated. Qualitative analysis of AFB1 using thin-layer chromatography (TLC) was performed .Ten microliters of the extract was applied on the TLC plate coated with Silica gel. Chloroform : Acetone (85:15) was used as the solvent system [11]. The plate was observed with a UV illuminator.

Degradation studies of AFB by LAB

10 mL of the MRS medium (pH 6.5) were added in tubes and inoculated with the Lactic Acid Bacterial strains and the separated aflatoxin (AFB1). The tubes were incubated for 48 h at 30°C. MRS medium inoculated with AFB1 toxin and none inoculated medium were served as control. The tubes were analysed in the same conditions.

Quantification of aflatoxin detoxification by the LAB isolates using HPLC

Determination of AFB1 content in MRS after fermentation was carried on by the method described by Gizzarelli *et al*. [12]. The extract was evaporated to dryness by using an evaporator system and is dissolved with 100 µ L of chloroform. The sample injection volume was set to 10 µL. The filtered and degassed solvents consisted of water (70%) and methanol (30%) will act as the mobile

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phase. The flow rate of the sample was 1mL/min. The retention time of AFB1 was approximately 7.2 min. The percentage of AFB1 removed from MRS broth by Lactic acid Bacterial isolates was calculated by the following formula:

$$\%\ of\ AFB1 = 100 \times [1 - (AFB1\ peakarea\ of\ the\ sample \div AFB1\ peakarea\ of\ the\ control)]$$

RESULTS AND DISCUSSION

Isolation and identification of fungi

Several strategies have been reported for the elimination or inactivation of mycotoxins. But only very few of the strategies such as ammonia treatment were accepted for practical use, and none is found to be effective completely. The biological degradation by using selected microorganisms should be the best approach for decontamination of mycotoxins. The toxigenic strain of *A. flavus*, used in the present study to produce AFB1, was isolated from the coconut and grape. The isolate was confirmed as *A. flavus* based on its colony appearance, morphological characteristics, and conidial arrangement (photo 1).

Isolation and Bacteriological analysis of the LAB

The biochemical results confirmed that all the three isolated strains were Gram positive and non-motile.

The strain showed no catalase activity. In the 0.3% methylene blue test that is used to distinguish *S. lactis* from *S. cremoris*, the former organism showed no growth but good growth was observed in latter one. All the strains showed good growth in 4% NaCl, but no growth was observed in 6.5% NaCl. All the isolated strains were further confirmed by sugar tests and the results are presented in Table 1 & 2. The results indicate that *S. lactis* was found to be positive for glucose, lactose, maltose, sucrose, galactose and fructose, mannitol was found to be negative. The results for *S. cremoris* specified that the strains showed positive reactions for lactose, glucose, galactose and fructose while gave negative reactions with sucrose, maltose, and mannitol. *Lactobacillus acidophilus* showed positive reactions with glucose, lactose, maltose, sucrose, galactose and fructose and showed negative reactions with mannitol. The results were compared with the Bergey's manual of determinative bacteriology and are found to be similar (Anonymous, 1974). From the biochemical characaterisation of the isolates it is evident to confirm the isolates as *S. lactis, S. cremoris* and *L. acidophilis.*

Screening of aflatoxin production

The isolates were screened for the production of AFB by being grown in YES medium for 7 days. The isolates capable of aflatoxin-production appeared as gray or black colonies under UV illumination, whereas non producing isolates appeared as white colonies. Aflatoxins B1 and G1 were responsible for the absorbance of UV light. The methodology carried on for the detection of aflatoxin by fluorescence of agar medium under ultraviolet light proves to be a simple and reliable means of eliminating non-producing strains and is also helpful to overcome the difficulties encountered in aflatoxin extraction from complex natural substrates[10]. In the present study detection of aflatoxin producing fungi is specified by the fluorescence upon exposure to UV radiation while growing on Hara et al., medium. The blue fluorescence and the appearance of gray or black colonies in the UV photographs confirm the presence of the aflatoxin production (photo 2).

Degradation studies of AFB by LAB

The different bacterial cell wall and cell envelope structures are probably responsible for the differences in aflatoxin binding by the strains [13]. In the present work the performance of *L.acidophilus* has been moderate with respect to other Lactic Acid Bacterial isolates. The high level of AFB1 can be responsible to inhibit the reduction potential of *L. acidophilus.* Among the investigated isolates *S. lactis* still retained in its position as the best detoxifying LAB (Fig 1). The best organism that could be recommended based on the total AFB1 reduced in this study was *S. lactis*, followed by *S. cremoris* and *L. acidophilus.* From the current study, it can be concluded that LAB attributes the antifungal potentiality and are effective food-grade biopreservatives for combating the problem of aflatoxin contamination. Therefore using of LAB for the detoxification strategy should be encouraged for its potential in reducing levels of toxic doses of aflatoxin along with its non toxic , non allergic

nature and health benefits.

Quantification of aflatoxin degradation by the LAB isolates using HPLC

The amount of toxin was measured using HPLC based on the retention time of the sample and it is evident that the concentration of Aflatoxin B1 during initial time was considerably higher than that found at the end of the incubation period along with LAB (Figure 1). It could also be confirmed that the rate of degradation of the obtained toxin varied with the type of LAB isolates. A subsequent HPLC chromatogram at 360 nm confirmed that the AFB1 peak eluting at 7.19 min disappeared and the peaks were found to be absent and reduced after incubation with the LAB.

This showed that AFB 1 is bio-transformed to other compounds [14]. In the present study the peak eluting at 7.28 confirms the presence of AFB1. The reduction in the peak area after incubation with LAB bacteria confirms the biotransformation of toxin by the action of LAB. The ability of lactic acid bacteria strains to reduce AFB1 after fermentation ranged from 56%-94% (Table 3). While the highest degradation was observed using *S. lactis and S. cremoris* in the *Aspergillus flavus* isolated from grapes, when compared to the *Aspergillus flavus* isolated from coconut. The results from TLC and HPLC analysis confirmed AFB1 bio-transformation and detoxification by LAB isolates.

CONCLUSION

The effect of LAB isolates on the carcinogenic and mutagenic aflatoxin B reduction was investigated. The results showed that *S. lactis* bind considerable amounts of the aflatoxin while compared to other *Lactobacillus* strains used in this work. The method investigated and developed in this study is expected to be useful for future researches in this area. The future trends are to include beneficial microorganisms like LAB in the detoxification of contaminated dietary foods and to constitute an approach for reduction of the availability of aflatoxins in the human diet and animal feed.

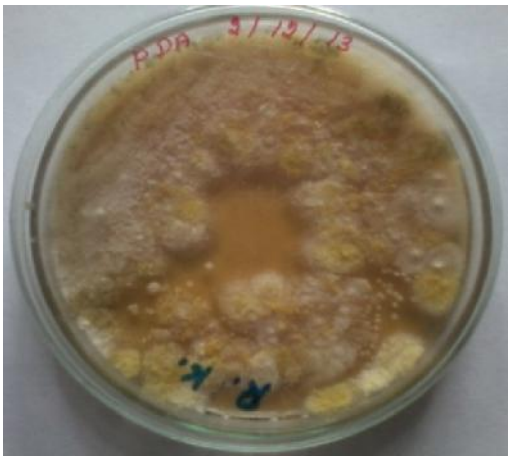

Photo 1. *Aspergillus flavus* in PDA plate

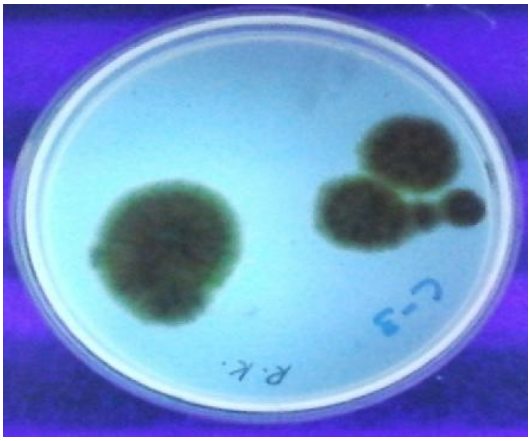

Photo 2. Blue fluorescence around the fungal colonies on UV exposure in the Hara *et al.,* medium.

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Table 1 - Biochemical and physiological properties of Lactic acid producing Bacteria

Culture	LAB1	LAB2	LAB3	LAB4	LAB5	LAB6	LAB7
Gram's staining	+	+	+	+	+	+	+
Morphology	Cocci in Chains	Rods	Cocci in Chains	**R**ods	rods	Cocci in Chains	Cocci in Chains
Catalase test	_	_	_	_	_	_	_
Motility	_	_	_	_	_	_	_
Growth on 4% Nacl	+	+	++	+	+	++	+
Growth on 6.5% Nacl	-	+	-	+	+	-	_
Growth at 10oC	_	_	+	+	_	+	_
Growth at 45oC	+	+	+	+	+	+	+
Growth on0.3%methylene blue	_	+	_	+	+	_	_
Isolate identified as	***Streptococcus lactis***	***Lactobacillus acidophilus***	***Streptococcus cremoris***	***Lactobacillus acidophilus***	***Lactobacillus acidophilus***	***Streptococcus cremoris***	***Streptococcus lactis***

Positive result = (+) Negative result = (-)

Table 2- Sugar fermentation properties of Lactic acid bacteria

Culture	Glucose	Sucrose	Lactose	Maltose
LAB1	+	+	+	+
LAB2	+	+	+	+
LAB3	+	+	+	-
LAB4	+	+	+	+
LAB5	+	+	+	+
LAB6	+	+	+	-
LAB7	+	+	+	+

Acid production = (+) No acid production = (-)

Table 3- HPLC analysis of the Aflatoxin and their relative percentage of biotransformation

S. No.	Name of the isolate	Retention time of Aflatoxin	Height [Cts]	Area	% of degradation
1.	Control	7.280	15.43	0.93	-
2	*S.lactis(1)*	6.832	9.2	0.06	56
3.	*S.cremoris(1)*	7.188	1	0.09	90.3
4.	*S.cremoris(2)*	7.009	1	0.06	94
5.	*S.lactis(2)*	7.368	1	0.06	94

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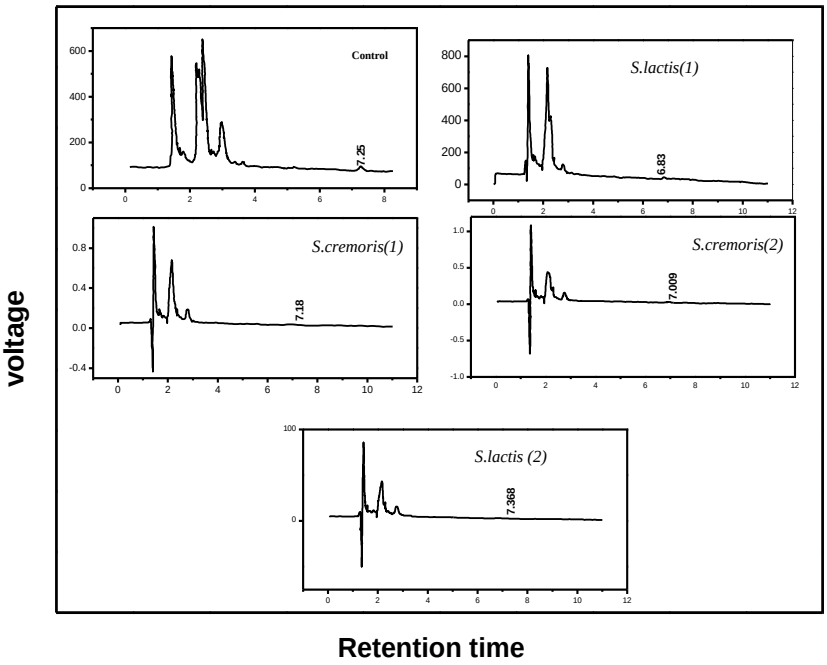

Fig 1. HPLC analysis of the aflatoxin

REFERENCES

[1] Leontopoulos D., Siafaka A., Markaki P., Black (2003) Olives as substrate for *Aspergillus parasiticus* growth and aflatoxin B1 production. Food Microbiol, 20:119-126.

[2] IARC, International Agency for Research on Cancer. (1993) Monograph on the Evaluation of Carcinogenic Risk to Human. vol. 56, pp. 257 – 263. Lyon, France.

[3] Meissonnier G.M.,Pinton P., Laffitte J., Cossalter A.M., Gong Y.Y., Wild C.P., Bertin G., Galtier P., Oswald I.P. (2008) Immunotoxicity of aflatoxin B1: impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicol. Appl. Pharmacol, 231142-149.

[4] Khlangwiset P., Shephard G.S., Wu F. (2011) Aflatoxins and growth impairment: a review. Crit. Rev. Toxicol, 41: 740-755.

[5] Swenson D.H., Lin J.K., Miller E.C., Miller J.A. (1977) Aflatoxin-2-3 oxide as a possible intermediate in the covalent binding of aflatoxin B1 and B to rat liver DNA and ribosomal RNA in vivo. Cancer Res, 37: 172-181.

[6] El-Nezamin H., Kankaanpaa P., Salminen S., Ahokas J. (1998) Physico-chemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media. J Food Prot, 61:466–68.

[7] Franz C.M.A.P., Cho G., Holzapfel W.H., Gálvez A. (2010) Safety of lactic acid bacteria. In: Mozzi F., Raya R., Vignolo G. (Eds.), Biotechnology of Lactic Acid Bacteria Novel Applications. Wiley-Blackwell, Iowa, USA.

[8] Shetty P.H. & Jespersen.L. (2006) *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents,Trends in Food Science & Technology,17: 48-55.

[9] Vosough M., Bayat M., and Salemi A. (2010) Matrix-free analysis of aflatoxins in pistachio nuts using parallel factor modeling of liquid chromatography diode array detection data. Analytica chimica acta, 663(1): 11–18.

[10] Hara S., Fennell D.I., Hesseltine C.W. (1974) Aflatoxin - producing strains of *Aspergillus flavus* detected by fluorescence of Agar medium under ultraviolet light. Applied Microbiology, 27(6):1118–1123.

[11] Horwitz W. (1975) Identification of aflatoxin by derivative formation on TLC plate. J. Assoc. Off. Anal. Chem, 12- 14.

[12] Gizzarelli S., L. Croci, L. Toti. (1993) Effect of lactic acid bacteria on aflatoxin production by *Aspergillus parasiticus* in laboratory conditions. Microbiol Alim Nutr, 11: 187–93.

[13] Haskard C.A., El-Nezami H.S., Kankaanaa P.E., Salminen S., Ahohas J.T. (2001) Surface binding of aflatoxin B1 by lactic acid bacteria. Applied Environmental Microbiology, 67: 3086–91.

[14] Melvin S., Samuel, Akella Sivaramakrishna, Alka Mehta (2014) Degradation and detoxification of aflatoxin B1 by *Pseudomonas putida*. International Biodeterioration & Biodegradation, 86: 202-209.